



Certification of a reference material consisting of genomic DNA inserts of *Bacillus licheniformis* DSM 5749 for PFGE IRMM-311

N. Meeus, L. De Baets, P. van Iwaarden, W. Philipp, H. Schimmel



EUR 23371 EN - 2008

The mission of the IRMM is to promote a common and reliable European measurement system in support of EU policies.

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements

Contact information

Address: Retieseweg 111, 2440 Geel, Belgium
E-mail: jrc-irrm-rm-sales@ec.europa.eu
Tel.: 014/571 705
Fax: 014/590 406

<http://irrm.jrc.ec.europa.eu/>
<http://www.jrc.ec.europa.eu/>

Legal Notice

Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use which might be made of this publication.

***Europe Direct is a service to help you find answers
to your questions about the European Union***

Freephone number (*):

00 800 6 7 8 9 10 11

(*) Certain mobile telephone operators do not allow access to 00 800 numbers or these calls may be billed.

A great deal of additional information on the European Union is available on the Internet.
It can be accessed through the Europa server <http://europa.eu/>

EUR 23371 EN
ISBN 978-92-79-09025-7
ISSN 1018-5593
DOI 10.2787/51728

Luxembourg: Office for Official Publications of the European Communities

© European Communities, 2008

Printed in Belgium

European Commission

IRMM information
REFERENCE MATERIALS

**Certification of a reference material
consisting of genomic DNA inserts of
Bacillus licheniformis DSM 5749 for PFGE**

IRMM-311

N. Meeus, L. De Baets, P. van Iwaarden, W. Philipp, H. Schimmel

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements (IRMM)
Geel, Belgium

Abstract

This report describes the production and certification of IRMM-311, a reference material of genomic DNA (gDNA) of *Bacillus licheniformis* DSM 5749 in agarose inserts. This CRM (IRMM-311) is intended to be used for the taxonomic identification of authorised probiotic feed additives by Pulsed Field Gel Electrophoresis (PFGE). The homogeneity and stability of the batch was assessed by monitoring the PFGE pattern of a *Sfi*I restriction enzyme digest of gDNA of *Bacillus licheniformis* DSM 5749 in agarose inserts. The batch was found to be homogeneous and the material is stable at 4 °C. The batch was characterised by five laboratories determining the PFGE pattern of a *Sfi*I restriction enzyme digest of IRMM-311. The pattern should be considered as a whole and restriction fragments in the size range from 50 kb to 90 kb are certified for their fragment length. DNA sequence analysis of the 3' end of the 16S rRNA gene and the entire 16S-23S internal transcribed spacer (ITS) coding region confirmed the identity of the strain as *Bacillus licheniformis*. Each vial contains one agarose insert containing gDNA of *Bacillus licheniformis* DSM 5749.

Table of contents

GLOSSARY.....	4
1 INTRODUCTION	5
2 PARTICIPANTS	6
3 PROCESSING	7
4 PROCEDURES	9
5 HOMOGENEITY.....	11
6 STABILITY	13
6.1 SHORT-TERM STABILITY	13
6.2 LONG-TERM STABILITY	14
7 BATCH CHARACTERISATION.....	16
8 ADDITIONAL CHARACTERISATION.....	18
9 METROLOGICAL TRACEABILITY.....	19
10 CERTIFIED VALUE AND UNCERTAINTY.....	20
11 INSTRUCTIONS FOR USE	21
12 REFERENCES.....	23
ACKNOWLEDGEMENTS	24
ANNEX 1	25

Glossary

BLAST	Basic Local Alignment Search Tool
bp	base pair
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTCS	dye terminator cycle sequencing
EDTA	ethylenediaminetetraacetic acid
ES	0.5 mol/L EDTA pH 8.0, 1 % (m/v) sodium lauryl sarcosine
ESP	ES containing proteinase K at 1 mg/mL
gDNA	genomic DNA
ITS	internal transcribed spacer
kb	kilo base pairs
LMP	low melting point
LTS	long-term stability
<i>N</i>	number of vials
<i>n</i>	number of replicates
n.a.	not applicable
no.	number
NB	nutrient broth
OD ₆₀₀	optical density at a wavelength of 600 nm
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PMSF	phenylmethanesulfonyl fluoride
rDNA	ribosomal DNA, DNA sequences encoding ribosomal RNA
RE	restriction enzyme
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RSD	relative standard deviation
<i>s</i>	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
STS	short-term stability
TBE	89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L EDTA, pH 8.3
TE	10 mmol/L Tris, 1 mmol/L EDTA, at specified pH
Tris	tris(hydroxymethyl)aminomethane

1 Introduction

Probiotic micro-organisms authorised for use as feed additives are described in Commission Regulation (EC) No 2148/2004 [1]. Pulsed Field Gel Electrophoresis (PFGE) is a powerful molecular typing method which can be used to discriminate different strains of bacterial species [2]. Bacterial strains yielding the same PFGE patterns are considered to be the same strain [3]. This report describes the production and certification of a batch of 400 vials of a reference material composed of genomic DNA (gDNA) of *Bacillus licheniformis* DSM 5749 in agarose (IRMM-311), in support of EU legislation. Council Directive 70/524/EEC concerning additives in feed regulates the use of probiotic bacilli and Directive 93/113/EC regulates the marketing of micro-organisms and their preparations in animal nutrition. According to both Directives, the use of specific genera of bacteria as feed additives are approved. One of the officially approved probiotics is *Bacillus licheniformis* DSM 5749. The certification of IRMM-311 was performed according to IRMM Reference Materials Unit procedures applying a quality management system according to ISO Guide 34 [4 - 7]. Homogeneity and stability of the produced batch was analysed at IRMM. Short-term stability (STS) measurements were performed following incubation at 4 °C and 18 °C in the frame of the certification project. Long-term stability (LTS) measurements were performed following incubation at 4 °C. The batch was characterised by monitoring the PFGE pattern of a restriction enzyme (RE) digest of selected samples by IRMM and 4 different external laboratories. Identification of *Bacillus licheniformis* DSM 5749 was confirmed by DNA sequencing of the nucleotide sequences of the 3' end of the 16S rDNA and the 16S–23S internal transcribed spacer (ITS) [9].

2 Participants

Bacillus licheniformis strain DSM 5749 originates from the Chr. Hansen A/S culture collection (Hørsholm, DK).

The processing and certification of IRMM-311 was performed at IRMM.

Participants for the batch characterisation were:

- Chr. Hansen A/S, Hørsholm, DK.
- Animal Sciences Group, Lelystad, NL.
- Food and Consumer Product Safety Authority, Zutphen, NL.
- University of Helsinki, Helsinki, FI.
- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE.

A summary of the different steps in the processing and certification of IRMM-311 is given in Table 1.

Table 1. Summary of different steps in the production of IRMM-311.

Project phases	Time period
Processing	Nov. - Dec. 2005
Certification	Feb. - Aug. 2006
Certification report and certificate	Oct. 2006 – Aug 2007

3 Processing

IRMM-311 consists of single agarose inserts, prepared from cultured bacterial cells of *Bacillus licheniformis* DSM 5749, mixed with melted agarose, allowed to solidify using 100 μ L insert molds. Molecular biology grade (sterile, DNase and RNase free) consumables and biochemicals were used at all time.

The processing steps for the production of IRMM-311 were:

1. Start 10 mL overnight culture of *Bacillus licheniformis* DSM 5749 in Nutrient Broth (NB) at 30 °C.
2. Inoculate the overnight culture into 100 mL NB medium, preheated to 30 °C in a 1 L Erlenmeyer flask. Grow culture to OD₆₀₀ of 0.6 (optical path length 10 mm) at 30 °C. Add chloramphenicol to a final concentration of 180 μ g/mL and incubate further for 1 h at 30 °C with shaking at 200 rpm.
3. Chill the culture on ice for 10 min. Spin down the cells by centrifugation (5000 \times g) at 4 °C for 30 min. Resuspend the pellet in 10 mL solution A (1 mol/L NaCl, 50 mmol/L Tris-HCl pH 7.6). Centrifuge the cells at 5000 \times g at 4 °C for 30 min. Resuspend cells in 5 mL cold (4 °C) solution A.
4. Prepare a 1 % (m/v) solution of low melting point (LMP) agarose in 50 mmol/L Tris-HCl buffer (pH 7.6) and cool it to 42 °C.
5. Mix 500 μ L 1 % (m/v) LMP agarose solution with 500 μ L cell suspension by inverting the tube several times. Use 10 sets of 10 reusable plug molds (BioRad) and with a pipet fill each insert mold with 100 μ L of the suspension. Put the insert molds on ice or at 4 °C for 20 min.
6. Fill 50 mL Falcon tubes with 25 mL of solution B (50 mmol/L Tris-HCl pH 7.6, 100 mmol/L EDTA, 100 mmol/L NaCl, 2 % (v/v) lysozyme) per tube. Push 50 agarose inserts into each tube and incubate in a shaking water bath overnight at 50 °C with shaking at 50 rpm.
7. Add Proteinase K (Qiagen) to a final concentration of 0.5 mg/mL.
8. Incubate 24 h in a shaking water bath (50 rpm) at 50 °C.
9. Remove solution carefully, and add 25 mL solution C to each tube (50 mmol/L Tris-HCl pH 7.6, 100 mmol/L EDTA, 100 mmol/L NaCl, 1 % SDS).
10. Incubate 24 h in a shaking water bath (50 rpm) at 50 °C.
11. Add RNase A (Qiagen) to a final concentration of 0,075 mg/mL, and incubate for another 24 h at 50 °C with shaking at 50 rpm.
12. Remove the solution, add 25 mL TE solution (10 mmol/L Tris-HCl pH 7.6, 1 mmol/L EDTA) containing 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), and incubate 1 h at room temperature. Repeat once.
13. Incubate inserts three times 30 min in TE solution at room temperature.
14. Label 100 plastic 3 mL vials (Nunc).

15. Transfer one agarose insert to each labeled plastic vial containing 1 mL TE.
16. Store the vials at 4 °C until digestion with REs.
17. Perform homogeneity and stability study and batch characterisation.

Steps 1 to 16 were repeated 4 times in order to produce 400 samples. As a control, PFGE analysis was performed on 2 random samples as described in section 4 and revealed the presence of intact high molecular mass DNA in the plugs.

4 Procedures

4.1 Minimum sample volume.

One agarose insert is used per analysis.

4.2 Digestion of agarose inserts with a RE

Digestion of agarose inserts with a RE was performed as described in section 11.2. More information on the procedure can be found in reference [8]. Four different REs (*NotI*, *SpeI*, *Ascl* and *SfiI*) were tested of which *SfiI* appeared to be the most suitable.

4.3 Pulsed Field Gel Electrophoresis.

The measurements in this report were performed using the Bio-Rad CHEF-DR® III System (Bio-Rad Laboratories, BE) using the method described in section 11.2. Other PFGE systems can be used as well. More information on the procedure can be found in reference [8].

4.4 16S-RNA strain identification and DNA sequencing.

16S-RNA strain identification was performed as described in [9]. Briefly, gDNA was isolated using the Qiagen genomic tip 20/G kit. 16S rRNA and 16S-23S ITS were amplified by PCR using primers L516SF and L523SR. Amplified fragments were visualised on agarose gels, extracted from gel using the Qiagen gel extraction kit and cloned into pCR2.1 (Invitrogen). Plasmids were purified using the QIAGEN Plasmid Mini Kit and sequenced using the dye terminator cycle sequencing (DTCS) reaction, according to the DTCS chemistry protocol [10]. Primers for sequencing were the universal M13 primers annealing to the 5' terminus of *lacZ*. Samples were analysed on a CEQ™ 8000 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA, US), using the following method: denaturation 120 s at 90 °C, injection 15 s at 2.0 kV, separation 85.0 min at 4.2 kV. As a control for the sequencing reaction the CEQ™ 8000 pUC18 plasmid was included on each sequencing plate. Validation of DNA sequencing using the CEQ™ 8000 system is described in detail in reference [11].

4.5 Sequencing of Pulse Marker

Pulse Marker is a mixture of λ DNA *Hind* III fragments, λ DNA, and λ concatemers embedded in 1% LMP agarose. The concatamers are two or more identical linear molecular units, covalently linked in tandem by means of single-stranded extensions or cos sites. In order to check the completeness of the concatemers of λ , i.e. no parts are missing at the covalent bond, the nucleotide sequence at the linking region of two DNA molecules in the marker was determined. A 1 mm slice of Pulse Marker D2291 (Sigma) was used as template in a PCR reaction amplifying a 216 base pair (bp) region including the end of 1 λ molecule and the start of a second. Based on the sequence in GenBank (J02459) primers were chosen 76 bp upstream and 87 bp downstream from the cos sites respectively (Figure 1). PCR was performed under

the following conditions: initial denaturation at 94 °C for 5 min was followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 20 s and 1 cycle of final elongation at 72 °C for 10 min. Amplified fragments were visualised on agarose gels, extracted from gel using the Qiagen gel extraction kit and cloned into pCR2.1 (Invitrogen). Plasmids were purified using the QIAprep spin Miniprep Kit and sequenced with M13 primers on an ABI Prism® 3130xl Genetic Analyser (Applied Biosystems, Lennik, BE) using the BigDye® Terminator v1.1 Cycle Sequencing kit.

```

48339 acgatacctg cgtcataatt gattatttga cgtgggttga tggcctccac gcacgttgtg
      tgctatggac gcagtattaa ctaataaaact gcaccaaact accggaggtg cgtgcaacac

48399 atatgtagat gataatcatt atcactttac gggtcctttc cggtgatccg acaggttacg
      tatacatcta ctattagtaa tagtgaaatg ccaggaag gccactaggc tgtccaatgc

1      gggcggcgac ctcgcgggtt ttcgctatct atgaaaattt tccggtttaa ggcgtttccg
      cccgccgctg gagcgcccaa aagcgataaa tactttttaa aggccaaatt ccgcaaaggc

61      ttcttcttcg tcataactta atgtttttat ttaaaatacc ctctgaaaag aaaggaaacg
      aagaagaagc agtattgaat tacaaaaata aattttatgg gagacttttc tttcctttgc

121     acaggtgctg aaagcgaggc tttttggcct ctgtcgtttc ctttctctgt ttttgtccgt
      tgtcacgac ttcgctccg aaaaaccgga gacagcaaag gaaagagaca aaaacaggca

```

Figure. 1: Nucleotide sequence of λ DNA (J02459) starting from base pair 48339 (end of first molecule) to base pair 171 (beginning of second molecule). Primers are indicated in bold upstream and downstream from the cos sites (highlight).

5 Homogeneity

The total number of samples produced was 400. The total number of samples to be used in the homogeneity study (N) was calculated as $\sqrt[3]{400} = 7.4$ [5]. In this study N is set to 8 and the number of replicates (n) is set to 1.

The homogeneity study was performed by monitoring the PFGE pattern of a *Sfi*I digest of the samples in comparison with commercial size standards for PFGE (D 2291 and D 2416, Sigma). Identical banding patterns were found for all 8 samples. Imaging was done using GeneSnap software and fragment length for restriction bands in the size range 50 kb – 90 kb were assigned using GeneTools software, copyright® of Synoptics Ltd., Cambridge, UK. The results of the homogeneity measurements are summarised in Figure 2 and Table 2.

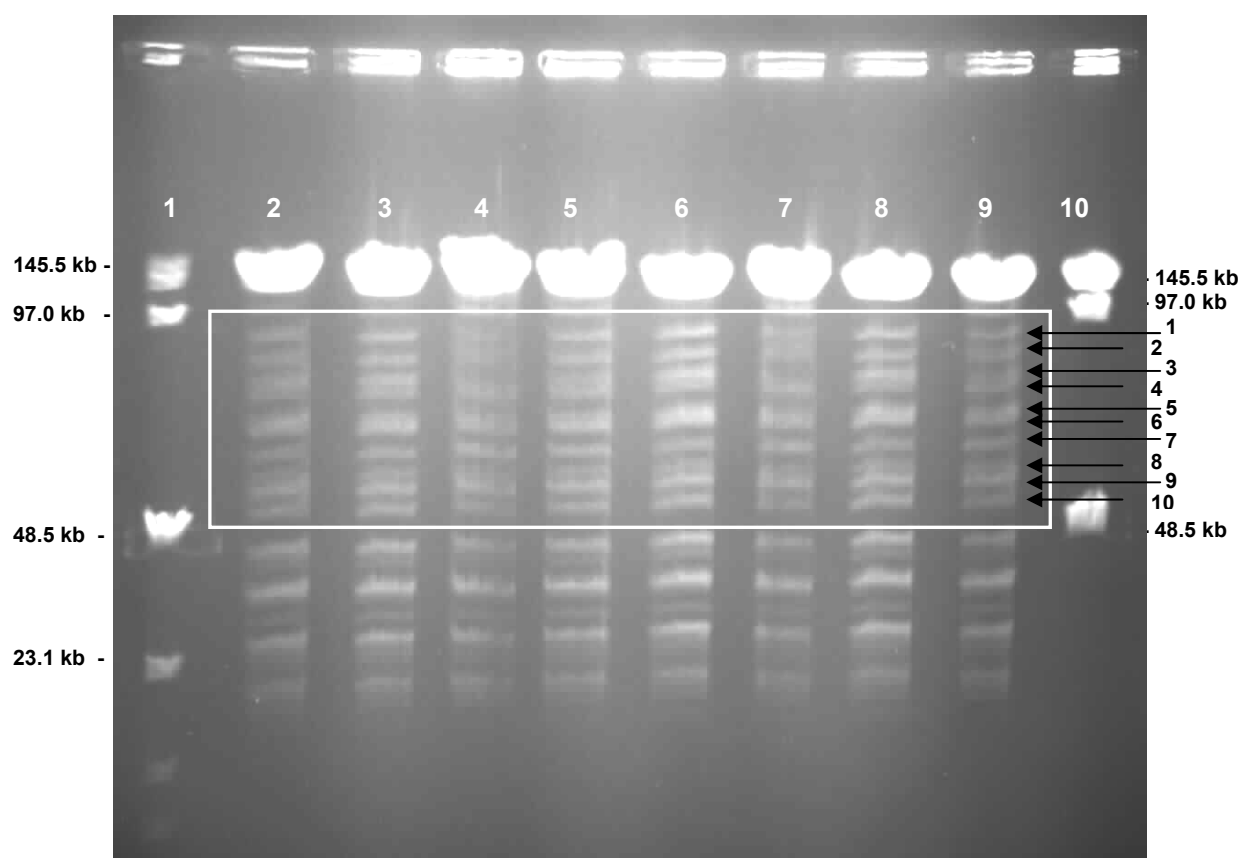


Figure 2. PFGE of *Sfi*I digest of gDNA of the samples used in homogeneity measurements of IRMM-311. Run conditions: initial switch time (= final switch time) = 3 s, run time = 20 h, 6 V per cm, angle = 120°, buffer temperature 14 °C. Lane 1: PFGE standard 0.1 - 200 kb (D 2291, Sigma); Lane 2-9: corresponds to the series of sample numbers in Table 2; Lane 10: PFGE standard 50 - 1000 kb (D 2416, Sigma). Restriction bands between 50 kb and 90 kb are marked from 1 to 10.

Table 2. Homogeneity of the PFGE pattern of IRMM-311. Assignment of fragment length [kb] to restriction bands between 50 kb and 90 kb of a *Sfi*I digest of each sample.

Sample Band no.	0032	0100	0139	0159	0250	0274	0338	0398	Fragment length [kb]
1	89.4	88.7	88.4	88.3	89.1	87.9	87.4	86.7	
2	81.9	82.2	82.4	81.8	82.8	83.1	81.4	81.5	
3	76.4	76.9	75.3	76.2	77.4	73.7	76.2	74.9	
4	73.7	73.7	73.2	73.5	75.7	73.0	73.4	73.3	
5	66.7	66.9	66.7	66.4	67.3	65.7	66.9	66.7	
6	65.9	65.9	65.9	65.4	66.7	65.1	65.8	65.3	
7	60.4	60.4	60.5	60.2	61.0	59.7	60.1	59.8	
8	55.8	56.4	54.4	56.0	55.6	55.6	55.6	54.0	
9	53.7	54.0	53.9	53.5	54.3	53.0	53.4	53.3	
10	50.2	50.4	50.3	49.8	50.4	49.2	49.6	49.6	

No suspicious data were detected based on the number and the positioning of the bands. No bands were missing and no new bands appeared. Therefore the complete set of data was used. No major differences in the intensity of the restriction patterns of the different samples was visually observed. Therefore the batch was found to be homogenous.

6 Stability

6.1 Short-term stability

The proper conditions for transport of the material to the customer were determined in a STS study consisting of isochronous incubation of samples at 4 °C and 18 °C for 0, 2 and 4 weeks. For each time/temperature combination two samples were tested. The PFGE pattern of a *Sfi*I digest of selected samples was analysed using commercial size standards.

The data from the STS study are summarised in Figure 3 and Table 3. No suspicious data were detected. Therefore, the whole data set was used. At 4 °C and 18 °C the material is stable, with all restriction bands between 50 kb and 90 kb detected by PFGE analysis. At 18 °C a slightly weaker signal is found, however all major bands are still visible. The material must be shipped at 4 °C (use of cooling elements). At this temperature, degradation of the material is negligible.

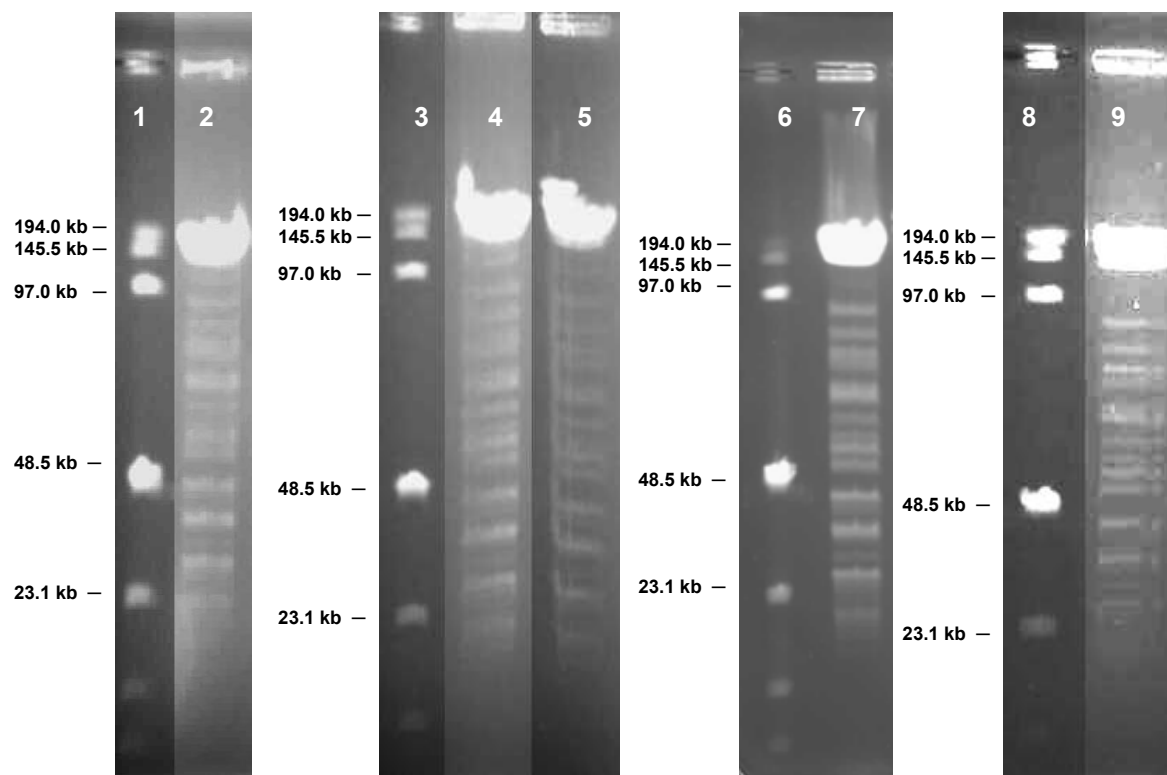


Figure 3. PFGE of *Sfi*I digests of the samples used for STS measurements of IRMM-311. Run conditions: initial switch time (= final switch time) = 3 s, run time = 20 h, 6 V per cm, angle = 120°, buffer temperature 14 °C. Lanes 1, 3, 6 and 8: D 2291 (Sigma); 2: 0004, 0 weeks; 4: 0087, 2 weeks, 4 °C; 5: 0058, 2 weeks 18 °C; 7: 0171, 4 weeks 4 °C; 9: 0135, 4 weeks, 18 °C

Table 3. STS of IRMM-311 stored at two different temperatures. At each time/temperature combination two different samples were tested. Assignment of fragment length (kb) of restriction bands between 50 kb and 90 kb of a *Sfi*I digest of each sample.

Temperature [°C]	n.a.		4				18			
Time [weeks]	0		2		4		2		4	
Sample	0004	0073	0087	0143	0171	0193	0058	0106	0135	0236
Band no.										
1	88.5	89.6	87.8	83.6	90.3	89.0	88.0	84.8	87.2	87.9
2	82.0	83.4	80.7	76.9	82.9	81.0	80.6	77.6	80.2	80.7
3	76.2	76.6	73.0	71.7	76.7	75.6	75.4	70.5	74.8	74.9
4	75.5	75.9	72.2	69.3	74.7	72.0	72.0	68.9	71.6	71.9
5	67.0	67.4	65.3	61.5	66.1	65.5	65.9	63.4	66.0	65.0
6	66.8	67.0	65.2	61.3	65.9	63.5	64.2	61.7	63.6	64.2
7	61.5	61.6	59.6	56.1	60.0	58.6	59.5	57.0	58.9	59.1
8	57.3	56.6	55.5	52.1	55.3	54.7	55.5	52.3	55.5	55.4
9	54.9	55.3	53.7	50.4	53.2	52.4	53.9	51.3	52.9	53.2
10	52.4	51.7	50.9	47.1	50.2	49.3	50.4	47.9	49.9	50.1

Fragment length [kb]

6.2 Long-term stability

The stability of the material during storage was determined from LTS studies. An isochronous study was carried out at 4 °C with time points of 0, 3, 6 and 12 months. Two samples ($n = 1$) per time point were tested. The PFGE pattern of a *Sfi*I digest of selected samples was analysed using commercial size standards. No spurious results were visible. Therefore, the whole data set was used. At 4 °C the material is stable, with all restriction bands between 50 kb and 90 kb detected by PFGE analysis. The data from the LTS study are summarised in Figure 4 and Table 4.

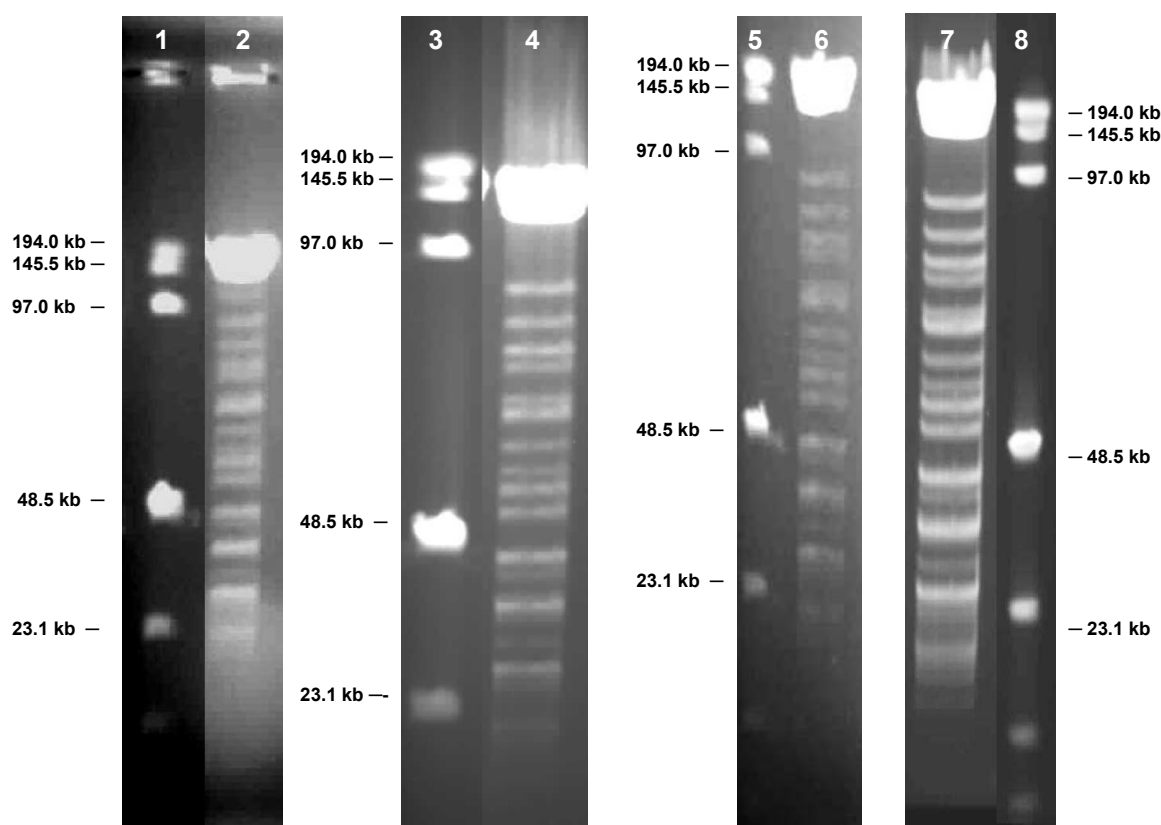


Figure 4. PFGE of *Sfi*I digest of gDNA of the samples used in the LTS measurements of IRMM-311. Run conditions: initial switch time (= final switch time) = 3 s, run time = 20 h, 6 V per cm, angle = 120°, buffer temperature 14 °C. Lanes 1, 3, 5 and 8: D 2291 (Sigma); Lane 2: 0073, 0

months 4 °C; Lane 4: 0195, 3 months 4 °C; Lane 6: 0239, 6 months 4 °C; Lane 7: 0319, 12 months 4 °C.

Table 4. LTS of IRMM-311 stored at 4 °C. At each time/temperature combination two samples were tested. Assignment of fragment length (kb) to restriction bands between 50 kb and 90 kb of a *Sfi*I digest of each sample.

Temperature [°C]	n.a.		4 °C					
Time [months]	0		3		6		12	
Sample Band no.	0004	0073	0126	0195	0239	0279	0319	0377
1	88.5	89.6	87.7	86.7	86.8	87.8	84.6	85.5
2	82.0	83.4	80.6	79.7	78.4	80.0	78.4	78.8
3	76.2	76.6	75.6	74.2	72.2	74.6	74.2	74.6
4	75.5	75.9	72.3	71.1	68.5	71.8	71.8	72.0
5	67.0	67.4	66.5	65.4	60.9	63.0	66.8	66.9
6	66.8	67.0	64.4	63.3	59.5	62.8	65.3	65.5
7	61.5	61.6	59.3	58.2	53.7	57.4	60.2	60.6
8	57.3	56.6	55.4	54.9	49.7	53.8	56.3	56.7
9	54.9	55.3	53.1	52.3	46.8	50.6	53.6	53.8
10	52.4	51.7	49.9	49.4	43.5	47.5	50.2	50.5

Fragment length [kb]

7 Batch characterisation

The batch of IRMM-311 was characterised by IRMM and four external laboratories. Each laboratory analysed two samples of IRMM-311 by PFGE following digestion with *Sfi*I according to the protocol in section 11.2 and reported the image of the PFGE gel. Fragment lengths of the characteristic restriction bands in the region between 50 kb and 90 kb were assigned using an appropriate software. The results are summarised in Table 5, Figure 5 and annex 1. Although not all gels are of the same quality, it is clear that all labs obtained an identical banding pattern.

Table 5. Batch characterisation of IRMM-311. Two samples were tested by each participant. Assignment of fragment length (kb) of restriction bands between 50 kb and 90 kb of a *Sfi*I digest. Mean of laboratory means, standard deviation (s) and relative standard deviation (RSD) are indicated.

Laboratory code		A		B		C	
Band no.	sample	0021	0060	0085	0109	345	387
1		89.7	89.0	95.6	96.0	88.2	88.1
2		83.2	82.6	81.1	81.0	80.5	80.2
3		78.1	78.0	73.1	73.3	75.5	75.2
4		74.8	75.1	68.5	68.2	72	72.4
5		69.0	69.4	65.6	65.4	66.9	67.1
6		66.9	67.3	61.3	60.8	64.4	64.8
7		61.7	61.8	59.4	59.2	59.3	59.7
8		57.9	58.3	55.9	55.4	55.6	56.1
9		54.8	55.6	53.2	52.4	52.9	53.3
10		51.4	51.9	50.4	49.8	48.9	49.6
Laboratory code		D		E			
Band no.	sample	0276	0320	0159	0250	mean	s
							RSD [%]
1		85.9	85.7	88.3	89.1	89.6	3.5
2		77.8	77.9	81.8	82.8	80.9	1.9
3		73.2	73.3	76.2	77.4	75.3	2.0
4		70.8	70.5	73.5	75.7	72.2	2.7
5		65.9	65.7	66.4	67.3	66.9	1.4
6		64	64	65.4	66.7	64.6	2.2
7		60.4	60.3	60.2	61.0	60.3	1.0
8		57.3	56.9	56.0	55.6	56.5	1.0
9		54.7	54.6	53.5	54.3	53.9	1.0
10		51.7	51.7	49.8	50.4	50.6	1.1

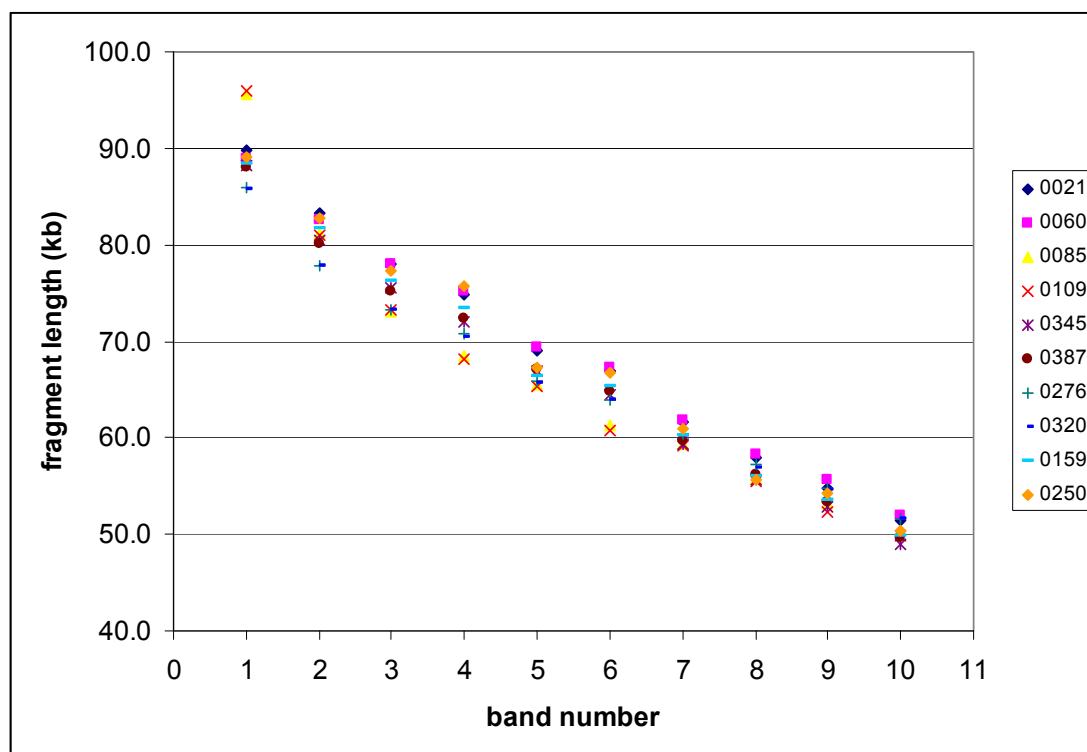



Figure 5. Results of batch characterisation. Graphical representation of fragment length of 10 restriction bands between 50 kb and 90 kb. Each symbol corresponds to a different sample analysed by one of the participating laboratories.

8 Additional characterisation

Identification of *Bacillus licheniformis* was confirmed by DNA sequencing of the nucleotide sequences of the 3' end of the 16S rDNA and the 16S–23S ITS [9]. The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. Using nucleotide-nucleotide BLAST the obtained sequence was compared to other nucleotide sequences in the GenBank database and showed 99 % homology with the reported DNA sequence of the 16S rDNA and the 16S–23S ITS of different strains of *Bacillus licheniformis* (Figure 6). For DSM 5749 this sequence is not published in the GenBank. Therefore a match of 100% was not found.

[gi|56160984|gb|CP000002.2|](https://www.ncbi.nlm.nih.gov/nuclotide/56160984)  *Bacillus licheniformis* ATCC 14580, complete genome
Length=4222334

Features in this part of subject sequence:

[rRNA-23S ribosomal RNA](#)

[rRNA-16S ribosomal RNA](#)

Score = 852 bits (430), Expect = 0.0
Identities = 442/446 (99%), Gaps = 0/446 (0%)
Strand=Plus/Minus

```

Query 77      CTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC 136
                |||
Sbjct 3121633 CTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC 3121574

Query 137     CGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCC 196
                |||
Sbjct 3121573 CGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCC 3121514

Query 197     GCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTG 256
                |||
Sbjct 3121513 GCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTG 3121454

Query 257     CGGCTGGATCACCTCCTTTCTAAGGATATTATACGGAATATAAGACCTTGCGGTCTTATA 316
                |||
Sbjct 3121453 CGGCTGGATCACCTCCTTTCTAAGGATATTATACGGAATATAAGACCTTGCGGTCTTATA 3121394

Query 317     GACAGGTACGTTTAGATCTTGTTTAGTTTGAAGGATCATTCCTTCAAAGCGTGTCTTT 376
                |||
Sbjct 3121393 GACAGGTACGTTTAGATCTTGTTTAGTTTGAAGGATCATTCCTTCAAAGCGTGTCTTT 3121334

Query 377     GAAAACTAGATAACAGTAGACATCACATTCAATTTCGTAATGCAAGATATCACGTAGTGAT 436
                |||
Sbjct 3121333 GAAAACTAGATAACAGTAGACATCACATTCAATTAGTAATGCAAGATATCACGTAGTGAT 3121274

Query 437     TCTTTTAAACGGTTAAGTTAGAAAGGGCGCACGGTGATGCCTTGGCACTAGGAGCCGAT 496
                |||
Sbjct 3121273 TCTTTTAAACGGTTAAGTTAGAAAGGGCGCACGGTGATGCCTTGGCACTAGGAGCCGAT 3121214

Query 497     GAAGGACGGGACTAACACCGATATGC 522
                |||
Sbjct 3121213 GAAGGACGGGACGAACACCGATATGC 3121188

```

Figure 6. Comparison of the homology of the nucleotide sequences of the 3' end of the 16S rDNA and the 16S–23S ITS obtained from IRMM-311 (Query) with the reported DNA sequence of the complete genome of *Bacillus licheniformis* ATCC 14580 in Genebank (accession no. CP000002), using nucleotide-nucleotide BLAST (blastn) on www.ncbi.nlm.nih.gov. Primers sites L516SF and L523SR are highlighted.

9 Metrological traceability

The obtained sequence of Pulse Marker showed 100 % homology with the λ sequence (J02459). No part was missing, no part was added and no repetitions were observed (Figure 7). We conclude that the marker consists of full λ concatamers. Therefore the fragment length of the *Sfi*I digested DNA fragments is traceable to intact λ DNA (isolated from the bacteriophage λ cl857 ind1 Sam7).

A.

ATTTGACGTGGTTTGGATGGCCTCCACGCACGTTGTGATATGTAGATGATAATCATTATCACTTTACGGGTCCTTTC
CGGTGATCCGACAGGTTACGGGGCGGCGACCTCGCGGGTTTTTCGCTATTTATGAAAATTTTCCGGTTTAAGGCGTT
TCCGTTCTTCTTCGTCATAACTTAATGTTTTTATTTAAATACCTCTGAAAAGAAAGGAAACG

B.

```

Score = 231 bits (120), Expect = 1e-57
Identities = 120/120 (100%), Gaps = 0/120 (0%)
Strand=Plus/Plus

Query 97  GGGCGGCGACCTCGCGGGTTTTTCGCTATTTATGAAAATTTTCCGGTTTAAGGCGTTTCCG 156
          |||
Sbjct 1    GGGCGGCGACCTCGCGGGTTTTTCGCTATTTATGAAAATTTTCCGGTTTAAGGCGTTTCCG 60

Query 157  TTCTTCTTCGTCATAACTTAATGTTTTTATTTAAATACCTCTGAAAAGAAAGGAAACG 216
          |||
Sbjct 61   TTCTTCTTCGTCATAACTTAATGTTTTTATTTAAATACCTCTGAAAAGAAAGGAAACG 120

Score = 185 bits (96), Expect = 7e-44
Identities = 96/96 (100%), Gaps = 0/96 (0%)
Strand=Plus/Plus

Query 1      ATTTGACGTGGTTTGGATGGCCTCCACGCACGTTGTGATATGTAGATGATAATCATTATCA 60
          |||
Sbjct 48407  ATTTGACGTGGTTTGGATGGCCTCCACGCACGTTGTGATATGTAGATGATAATCATTATCA 48466

Query 61     CTTTACGGGTCCTTTCCGGTGATCCGACAGGTTACG 96
          |||
Sbjct 48467  CTTTACGGGTCCTTTCCGGTGATCCGACAGGTTACG 48502

```

Figure. 7 A. Nucleotide sequence of Pulse Marker D 2291 obtained with M13 primers on an ABI Prism® 3130xl Genetic Analyser. The highlighted part is the start of the second λ molecule. B. Comparison of sequence identities between obtained sequence (A.) and λ sequence (J02459).

The identity of the IRMM-311 has been confirmed by dye terminator cycle sequencing of the nucleotide sequences of the 3' end of the 16S rDNA and the 16S–23S internal transcribed spacer (ITS) [9].

10 Certified value and uncertainty

The certified properties of IRMM-311 are the fragment lengths [kb] of 10 DNA fragments in a PFGE pattern as determined by each of five laboratories. The certified value for each band is the mean fragment length calculated from the means obtained by five different laboratories (Table 6). The PFGE pattern was obtained by *Sfi*I digestion of gDNA of *Bacillus licheniformis* DSM 5749 in agarose inserts (Figure 8). The obtained band pattern of an unknown sample should always be compared to that of the reference material on the same agarose gel, considering all *Sfi*I fragments in size between 50 kb and 90 kb. Uncertainties for the certified values were taken as the half-width of the 95 % confidence interval of the mean.

Table 6. Unweighted mean of the means of five accepted sets of results, obtained by a *Sfi*I digest of gDNA of *Bacillus licheniformis* DSM 5749.

IRMM-311: <i>Bacillus licheniformis</i> DSM 5749	
Certified value (kb)	Uncertainty (kb)
89.6	4.7
80.9	2.5
75.3	2.7
72.2	3.5
66.9	1.9
64.6	2.9
60.3	1.3
56.5	1.3
53.9	1.3
50.6	1.3

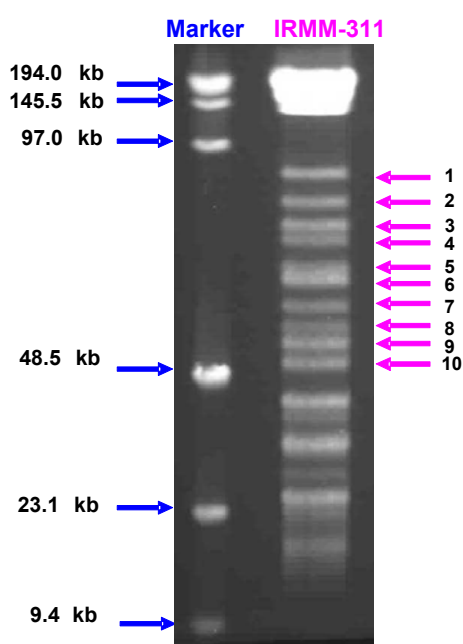


Figure 8. PFGE pattern of a *Sfi*I digest of gDNA of *Bacillus licheniformis* DSM 5749 in agarose inserts when analysed according to a specified procedure (see section 11). Restriction bands between 50 kb and 90 kb are marked from 1 to 10.

11 Instructions for use

11.1 Dispatch

Dispatch to the customer shall be done at 4 °C (use of cooling elements). Upon receipt by the customer, the material should be stored at 4 °C until use.

11.2 Handling and intended use

This CRM is intended to be used for the taxonomic identification of the authorised probiotic feed additive *Bacillus licheniformis* DSM 5749 by PFGE.

Molecular biology grade (sterile, DNase-, RNase-free) consumables and biochemicals shall be used in all procedures.

11.2.1 Digestion of agarose inserts with *Sfi*I restriction enzyme

- Transfer the agarose insert of IRMM-311 to a 1.5 mL tube containing 1 mL 10 mmol/L Tris-HCl buffer (pH 7.6) and incubate 30 min at room temperature.
- Remove the solution and add another 1 mL 10 mmol/L Tris-HCl buffer (pH 7.6), incubate again 30 min at room temperature.
- Remove solution without destroying the plug.
- Incubate insert two times 30 min at room temperature in 0.5 mL RE buffer for *Sfi*I (use RE and appropriate buffer from manufacturer of choice). Renew restriction buffer in between.
- Remove restriction buffer.
- Add to each insert: 30 µL 10 × concentrated RE buffer for *Sfi*I, 30 U *Sfi*I and add H₂O to a final volume of 300 µL.
- Incubate at 50 °C for 15 -17 h.
- Remove solution carefully, add 1 mL of ES solution (0.5 M EDTA pH 8.0, 1 % (w/v) sodium lauryl sarcosine).
- Incubate overnight at 50 °C.
- Remove solution, add 250 µL ESP (ES containing proteinase K at 1 mg/mL).
- Incubate 2 h at 50 °C.
- Dilute Tris-Borate-EDTA (TBE) buffer (89 mmol/L Tris-borate, 2 mmol/L EDTA, pH 8.3), e.g. Sigma T7527, with distilled water in a volume ratio 1:3 (buffer:water).
- Remove solution, add 1 mL diluted TBE buffer and incubate 1 h at room temperature. Inserts can be kept in this solution for several days at 4 °C.
- Take a 1 mm slice of agarose inserts of each of the PFGE standards D2291 and D2416 and incubate in 1 mL diluted TBE for 1 h at room temperature.

11.2.2. PFGE

For other systems than Bio-Rad CHEF-DR[®] III System (Bio-Rad Laboratories, BE) use the instructions of the manufacturer, applying run conditions for electrophoresis as described below.

- Prepare a 1.2 % gel of PFGE certified agarose using diluted TBE.
- Melt the agarose suspension.
- Assemble the casting stand and pour in the agarose solution, remove any air bubbles and place comb near one of the ends. Allow the gel to solidify for at least 30 min.
- Fill the chamber with 2,5 L diluted TBE.
- Start buffer pump and remove air bubbles trapped in the pump. Set running temperature at 14 °C and start cooler. Circulate buffer at least 20 min.
- Melt 0.8 % LMP agarose prepared in diluted TBE, and cool it to 45-50 °C.
- Remove the comb from the gel.
- Load the samples carefully into the slots. Load the 1 mm slice of size markers on both sides of the sample lanes.
- Remove excess buffer and seal the holes with 0.8 % LMP agarose in diluted TBE.
- Take gel and plate out of the casting stand. Remove any agarose which might stick underneath the plate.
- Place plate with gel in the frame in the electrophoresis chamber, and start run using the following conditions: initial switch time (= final switch time) = 3 s, run time = 20 h, 6 V per cm, angle = 120 °, buffer temperature 14 °C.
- After electrophoresis stain gels 30 min in 250 mL 1 µg/mL Ethidium Bromide in diluted TBE.
- Destain in 250 mL H₂O for 2 h.
- Photograph the gel over UV-source and assign fragment lengths to size markers. Standard Sigma D2291 contains the following size markers in the region of interest: 194, 145.5, 97, 48.5 (more concentrated band), 23.1 and 9.4 kb. Standard Sigma D2416 contains the following size markers in the region of interest: 194, 145.5, 97, 48.5 kb.
- Analyse data for sample tracks according to Gel Documentation System in place. Analyse pattern and fragment length of all bands between 50 kb and 90 kb.
- If necessary destain for additional 24 h in H₂O. The gel can be stored in diluted TBE at 4 °C, if necessary.

12 References

- [1] Commission Regulation (EC) No 2148/2004 of 16 December 2004 concerning the permanent and provisional authorisations of certain additives and the authorisation of new uses of an additive already authorised in feedingstuffs. Off. J. Eur. Commun. L 370 (2004), 24
- [2] Olive, D.M., Bean, P. (1999) Principles and Applications of Methods for DNA-Based Typing of Microbial Organisms. J. Clin. Microbiol. 37, 1661–1669.
- [3] Tenover, F.C., Arbeit R.D., Goering R.V., Mickelsen P.A., Murray B.E., Persing D.H., Swaminathan, B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33 (9), 2233-2239
- [4] ISO Guide 34: 2000, General requirements for the competence of reference material producers. Organization for International Standardization, Genève.
- [5] van der Veen, A.M.H., Linsinger, T.P.J., Pauwels, J. (2001) Uncertainty calculations in the certification of reference materials. 2. Homogeneity study. Accred. Qual. Assur. 6, 26-30.
- [6] van der Veen, A.M.H., Linsinger, T.P.J., Lamberty, A., Pauwels, J. (2001) Uncertainty calculations in the certification of reference materials. 3. Stability study. Accred. Qual. Assur. 6, 257-263.
- [7] van der Veen, A.M.H., Linsinger, T.P.J., Schimmel, H., Lamberty, A., Pauwels, J. (2001) Uncertainty calculations in the certification of reference materials 4. Characterisation and certification. Accred. Qual. Assur. 6, 290-294.
- [8] Sambrook, J. , Fritsch, E.F., Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, 2nd edition.
- [9] Xu, D., Côte, J-C. (2003) Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S–23S ITS nucleotide sequences. IJSEM, 53, 695–704.
- [10] CEQ 8000 Series – DTCS chemistry protocol. <http://www.beckmancoulter.com/Literature/BioResearch/390003ac.pdf>. Beckman Coulter, Inc. (2003).
- [11] Galvin, N.M., Clark, M.W., Reddy, M.P. (1998) CEQ™ 2000 DNA Analysis System Performance, Beckman Coulter, Inc.

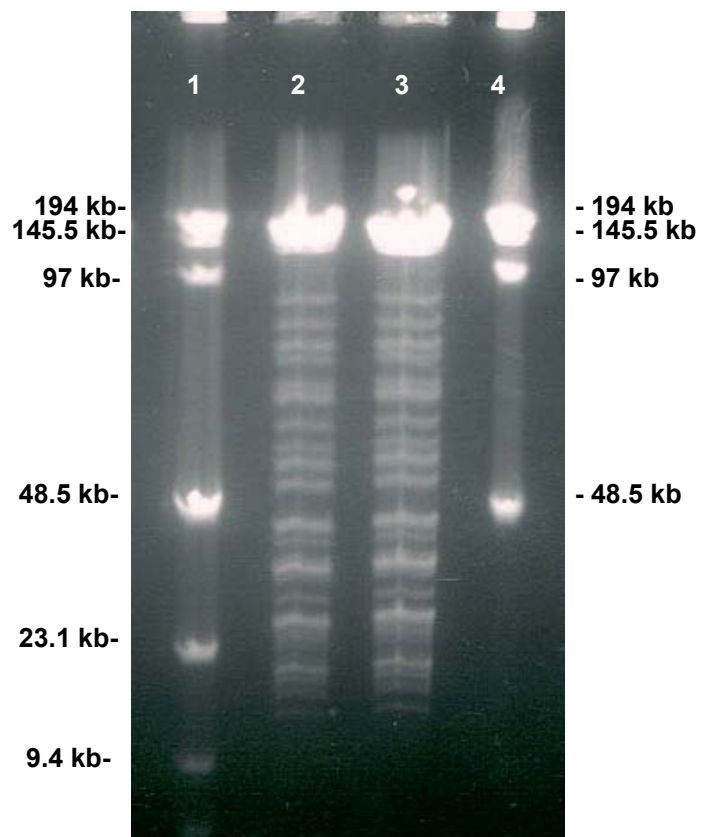
Acknowledgements

The authors would like to thank R. Zeleny and W. Broothaerts (IRMM) for reviewing the certification report, as well as the experts of the Certification Advisory Panel 'Biological Macromolecules and Biological/Biochemical Parameters', R. Dybkaer (Frederiksberg Hospital, DK), E. Jansen (National Institute of Public Health and the Environment, NL) and U. Örnemark (LGC Promochem, SE) for their critical comments.

Annex 1: IRMM-311 batch characterisation – results received from five laboratories

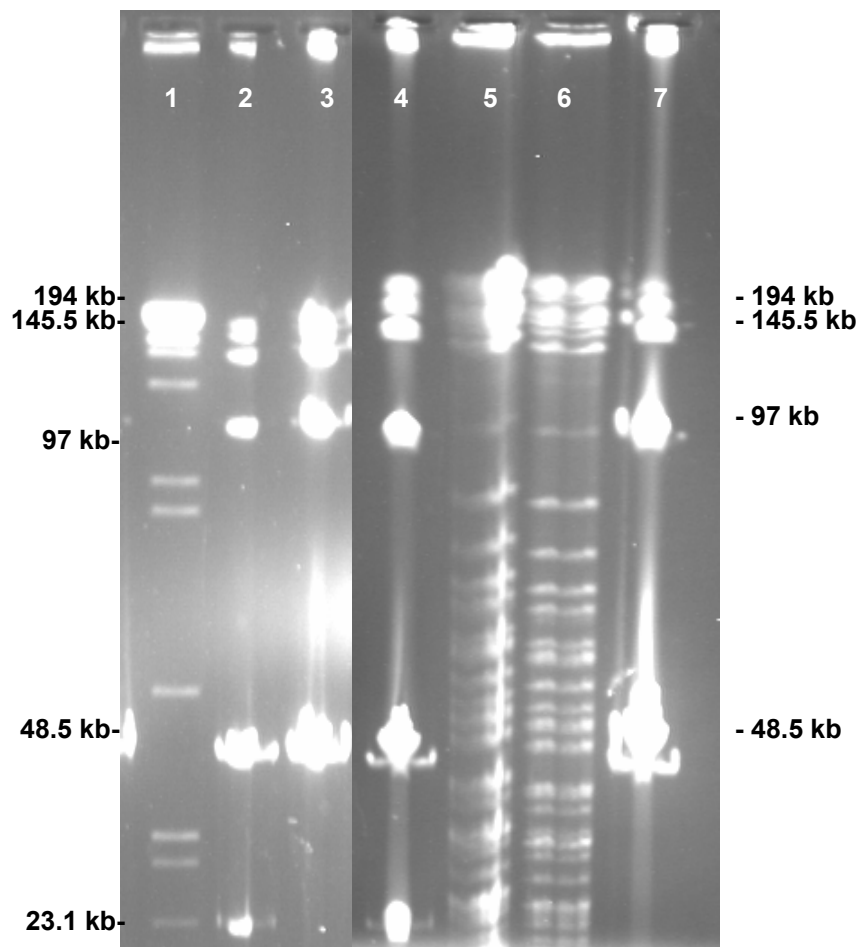
Laboratory A

1. D2291 marker
2. IRMM-311 # 0021
3. IRMM-311 # 0060
4. D2416 marker



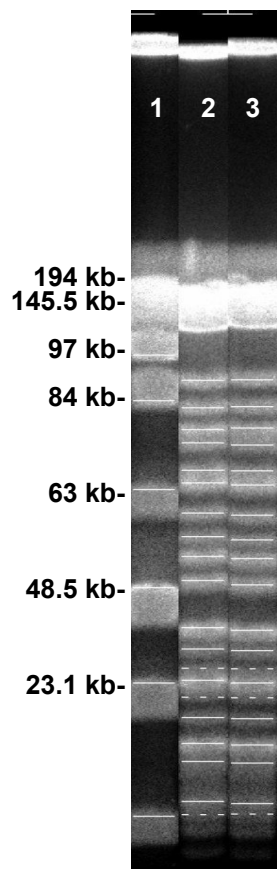
Laboratory B

1. In house Marker
2. D2291 marker
3. D2416 marker
4. D2291 marker
5. IRMM-311 # 0085
6. IRMM-311 # 0109
7. D2416 marker



Laboratory C

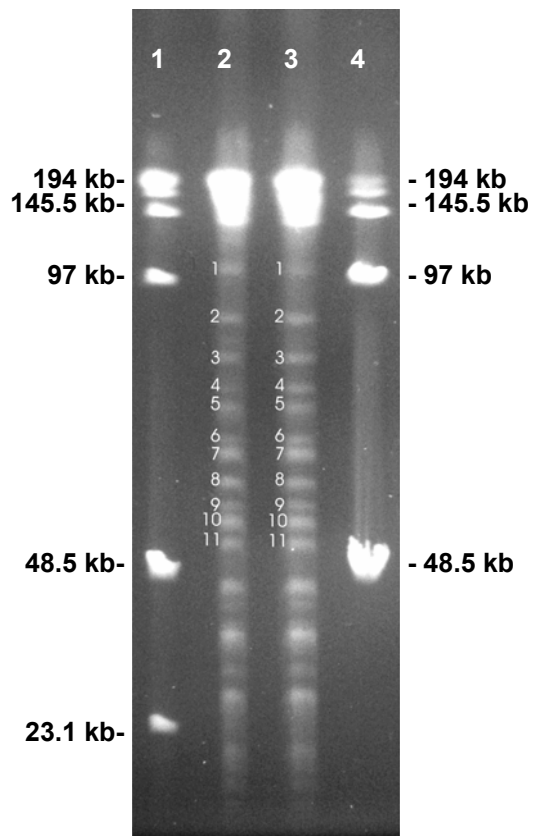
1. MidRange PFG Marker I, NEB N3551S
2. IRMM-311 # 0387
3. IRMM-311 # 0345



Laboratory D

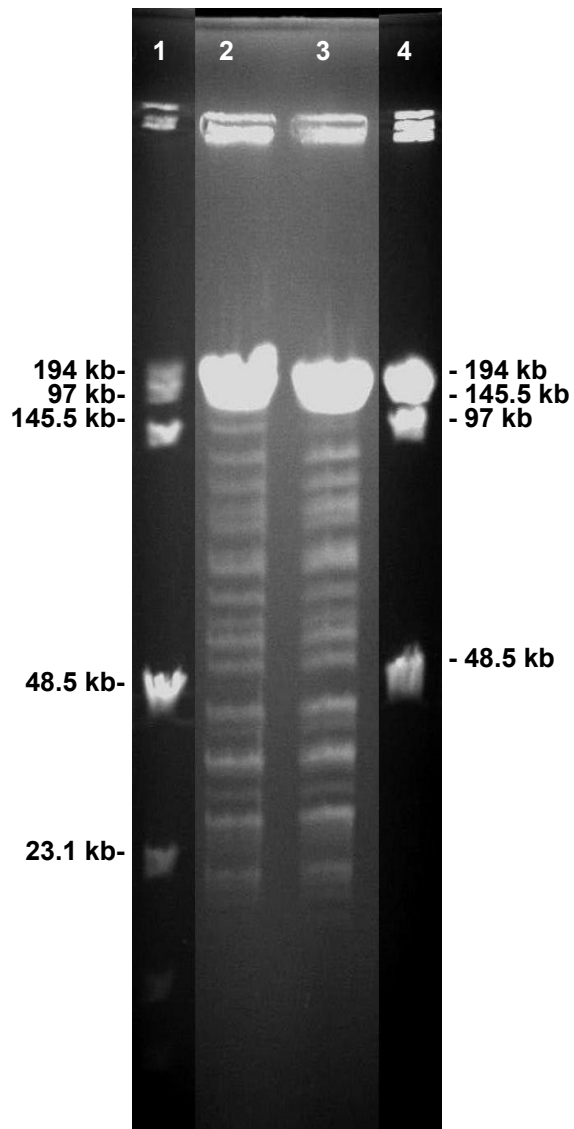
1. D2291 marker
2. IRMM-311 # 0276
3. IRMM-311 # 0320
4. D2416 marker

The band numbers on the gel should not be taken into account as they do not correspond to IRMM's assignment.



Laboratory E

1. D2291 marker
2. IRMM-311 # 0159
3. IRMM-311 # 0250
4. D2416 marker



European Commission

EUR 23371 EN – Joint Research Centre – Institute for Reference Materials and Measurements

Title: Certification of a reference material consisting of genomic DNA inserts of *Bacillus licheniformis* DSM 5749 for PFGE, IRMM-311

Authors: N. Meeus, L. De Baets, P. van Iwaarden, W. Philipp, H. Schimmel

Luxembourg: Office for Official Publications of the European Communities

2008 – 29 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1018-5593

ISBN 978-92-79-09025-7

DOI 10.2787/51728

Abstract

This report describes the production and certification of IRMM-311, a reference material of genomic DNA (gDNA) of *Bacillus licheniformis* DSM 5749 in agarose inserts. This CRM (IRMM-311) is intended to be used for the taxonomic identification of authorised probiotic feed additives by Pulsed Field Gel Electrophoresis (PFGE). The homogeneity and stability of the batch was assessed by monitoring the PFGE pattern of a *Sfi*I restriction enzyme digest of gDNA of *Bacillus licheniformis* DSM 5749 in agarose inserts. The batch was found to be homogeneous and the material is stable at 4 °C. The batch was characterised by five laboratories determining the PFGE pattern of a *Sfi*I restriction enzyme digest of IRMM-311. The pattern should be considered as a whole and restriction fragments in the size range from 50 kb to 90 kb are certified for their fragment length. DNA sequence analysis of the 3' end of the 16S rRNA gene and the entire 16S-23S internal transcribed spacer (ITS) coding region confirmed the identity of the strain as *Bacillus licheniformis*. Each vial contains one agarose insert containing gDNA of *Bacillus licheniformis* DSM 5749.

How to obtain EU publications

Our priced publications are available from EU Bookshop (<http://bookshop.europa.eu>), where you can place an order with the sales agent of your choice.

The Publications Office has a worldwide network of sales agents. You can obtain their contact details by sending a fax to (352) 29 29-42758.

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

